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We have recently identified a new human syndrome characterized by breast cancer, autoimmunity directed against the neuronal protein in amphiphysin, and Stiff-Man syndrome (SMS). SMS is a rare disease of the central nervous system characterized by progressive rigidity of the body musculature. This condition appears to represent a novel entity within the emerging family of neurological autoimmune paraneoplastic syndromes, conditions in which ectopic expression of a neuronal protein in cancer cells lead to autoimmunity and eventually to neurological disease. We are characterizing the role of amphiphysin and amphiphysin-related proteins in SMS and breast cancer patients. We expect that these studies will provide new information on autoimmune mechanisms in breast cancer, may help to develop new tools for the early diagnosis of this condition an may suggest new avenues for immunotherapy. In the last year, we have focused on three major areas: (1) we screened 54 patients and identified 2 new patients with autoimmunity to amphiphysin and cancer. In one of these patients screening for autoimmunity served as a diagnostic procedure for cancer. (2) we have cloned and characterized a splice variant of amphiphysin 1, p108, from a human breast cancer cell line. (3) We are studying the interaction of amphiphysin with p35, an activator of the neuronal kinase cdk5.

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PI - Signature Date

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INTRODUCTION

Stiff Man Syndrome (SMS) is a rare neurological disorder characterized by progressive rigidity of the body musculature with superimposed painful spasms. (Lorish, T.R., et al. Mayo Clin. Proc. 64:629-636, 1989). Recent studies suggest that SMS is an autoimmune disease (Folli, et. al. New Engl. J. Med. 328:546-551, 1993) In these studies, antibodies directed against synaptic proteins have were detected in the serum of patients with SMS. Two major target proteins for these antibodies have been identified: glutamic acid decarboxylase (GAD) and amphiphysin. Amphiphysin is a protein involved in endocytosis which is highly expressed in neural tissue (David, C. et. al., PNAS USA 93(1) 331-5, 1996). Although SMS patients with anti-amphiphysin antibodies are less common than those with anti-GAD antibodies (11 reported cases verses 100 cases), these cases deserve special attention in that all patients with amphiphysin auto-immunity have cancer, most commonly breast cancer. Indeed, in some cases the cancer was found only after the presence of anti-amphiphysin antibodies prompted an aggressive search. Endocytosis proteins have also recently received attention for roles in cell signaling and cell cycle control (Vieira, A.V., et. al., Science 274:2086-8, 1996; Sakamuro, D., et. al. Nature Genetics 14:69-76, 1996; Floyd and De Camilli, Trends in Cell Biology 8:299-301, 1998). Amphiphysin therefore appears to be a protein that is involved in cancer, autoimmune disease and the endocytotic function of the nervous system. We intend to identify new cases of autoimmunity to amphiphysin in the context of cancer, and to understand the relationships between cancer, the development of autoimmunity, and neurological disease. This research and study of the structure and function of amphiphysin and related proteins can lead to earlier detection of cancer, and may provide better understanding of some of the processes that lead to cancer. Through this research, we hope to improve diagnosis and treatment for the many people who suffer from these diseases.

BODY

Methods

We have used immunofluorescence labeling of rat brain sections with the serum and cerebral spinal fluid for screening of patients with neurological disease. Human sera and cerebral spinal fluid that are positive for auto antibodies to amphiphysin by immunofluorescence methods are then further tested by Western blot of brain extract. We have performed Western blotting of breast cancer cell lines and primary human tissues with amphiphysin antibodies developed in our lab to study amphiphysin expression in cancer. RT-PCR and screening of a phage library developed from a breast cancer cell line resulted in the identification of a non-neuronal amphiphysin isoform overexpressed in breast cancer. We are presently using in-vitro kinase assays and transfection experiments of human cells in culture to further investigate the biological role of amphiphysin especially as it relates to the growth and differentiation of human cells.

Results and Discussion

In the past year, our ongoing work to find patients with neurological disease, cancer and autoimmunity to amphiphysin has screened 54 patients and identified 2 new patients that have autoimmunity to amphiphysin and cancer. One of these patients showed the neurological features of Stiff-man Syndrome before a cancer was found. The patient was demonstrated to have auto antibodies against amphiphysin by our lab, and subsequently, a neoplastic lymph node was found. In this way, screening of this patient for autoimmunity served as a diagnostic procedure for cancer.

The connection of amphiphysin autoimmunity with cancer and the properties of amphiphysin II and RVS161/167 have prompted us to investigate the role of amphiphysin I in the biology of cancer. Work accomplished along these lines in the past year includes the identification and molecular cloning of a splice variant of amphiphysin I, p108, from a human breast cancer cell line. Using western blotting with antibodies developed in the lab, we showed that this isoform is expressed in a wide variety of non-neuronal tissues, and is overexpressed in two breast cancer cell lines and in several primary human breast tumors, including the tumor of a patient with a paraneoplastic neurological syndrome. We also showed that the SH3 domain of this amphiphysin I isoform interacts with the proline rich domains of dynamin I and synaptojanin I using affinity chromatography and recombinant proteins (Floyd et al. Molecular Medicine 4: 29-39 1998). In the cell line that expresses p108, a pool of this protein interacts with amphiphysin II isoforms as evidenced by co-immunoprecipitation experiments

Work in budding yeast systems have provided clues to the function of amphiphysin in mammalian cell. Other researches have outlined a role for amphiphysin in growth control and actin cytoskeleton dynamics in yeast. These findings are tantalizing when viewed in the context of a potential role for amphiphysin in cancer. One of the most recent findings from work in yeast demonstrated the interaction of an amphiphysin homologue, RVS 167 with the PHO85 kinase complex (Current Biology 24:1310-2,1998). The mammalian homologue of this kinase complex is the p35/cdk5 complex, which has a role in actin cytoskeletal dynamics and development of the nervous system. Our current work centers on the interaction of amphiphysin I with p35, an activator of the neuronal kinase cdk5. We have preliminary evidence that p35 binds to the N-terminal region of amphiphysin I and that amphiphysin I is a substrate for the kinase activity of cdk5. These proteins are also found together in the growth cones of neurons in culture. We also have preliminary evidence that an isoform of p35 is expressed in breast cancer cells and interacts with amphiphysin. These findings point to a potential role for amphiphysin and the p35/cdk5 complex in the function of neurons and mammary tissue. These findings will hopefully lead to a better understanding between cancers in mammary tissue and neurological dysfunction.

CONCLUSIONS

Amphiphysin is a target of autoimmunity in the setting of breast cancer. The presence of autoimmunity to amphiphysin is a diagnostic indicator to search for occult neoplasm in patients who present with neurological syndromes in the absence of cancer. Amphiphysin is aberrantly expressed in some breast cancers. Amphiphysin may play a role in general mechanisms of growth control and differentiation both in the nervous system, and in non-neuronal tissues such as mammary tissues. The aberrant expression of amphiphysin in these tissues may be involved in the genesis of cancer as well as in the autoimmune response.

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APPENDIX A
(Figure legends)

Fig. 1

Identification of anti-amphiphysin I autoimmunity in the serum of a patient with paraneoplastic sensory neuronopathy (patient #692). Panel A. Total rat brain homogenate was analyzed by western blotting with the following sera: (a) serum of the patient, (b) serum of a normal control patient, (c) rabbit serum CD9 that recognizes amphiphysin I and II (19). Panel B. The epitope specificity of the autoantibodies from patient #692 was mapped by western blot using GST fragments corresponding to the overlapping fragments of amphiphysin I depicted. Most autoantibodies reacted with fragment V, with a weaker response to fragment III. Electrophoretic mobility of the fragments is indicated at right.

Fig. 2

Detection of amphiphysin I immunoreactivity in the cancer tissue of patient #692. Total homogenates of human brain (a) and of the cancer tissue from patient #692 (b) were analyzed by western blotting using the anti-amphiphysin I rabbit serum CD5 as a probe. Brain contained a single 128 kDa amphiphysin I immunoreactive band (one asterisk), while the cancer tissue contained both this band and a lower immunoreactive band of about 108 kDa (two asterisks). Total protein loading was as follows: (a), 100 μgs; (b), 35 μgs.

Fig. 3

Expression of amphiphysin I immunoreactivity in human cell lines derived from normal and neoplastic breast tissue. Panel A: total protein homogenates of human brain (10 μgs) or of human cell lines (20 μgs) were probed by western blotting with the rabbit anti-amphiphysin I antiserum CD5. Lanes are as follows: (a) rat brain, (b) MDA-MB-453 breast cancer cell line, (c) MCF7 breast cancer cell line, (d), MDA-MB-231 breast cancer cell line, (e) Hs578T breast cancer cell line, (f) SK-BR3 breast cancer cell line, (g)

Hs578T cell line, (h) Hs578Bst breast tissue cell line. Panel B: western blotting with the CD5 antiserum of (a) the cancer tissue of patient #692, (b) the Hs578T cell line, and (c) a normal breast tissue cell line (MCF-10A) demonstrating the identical electrophoretic mobility of the 108 kDa bands in the cell line and in the cancer tissue. Panel C: total homogenates of (a) rat brain and of (b) the cell line Hs578T probed by western blotting with antibodies directed against the neuronal proteins indicated. 10 μg of rat brain and 20 μg of cell extract were loaded in (a) and (b) respectively. One and two asterisks point to the 128 and the 108 kDa bands respectively.

Fig. 4

Reactivity of the breast cancer tissue of patient #692 and of the cell line Hs578T with 5 monoclonal antibodies directed against 5 distinct regions of amphiphysin I. Total homogenate proteins (30 μgs for lanes a and 300 μgs for the other lanes) were reacted by western blotting with monoclonal antibodies directed against each of the 5 amphiphysin I fragments depicted in fig. 1B. Lanes are as follows: (a) human brain; (b) cancer tissue of patient #692; (c), cell line Hs578T; and (d) cell line MCF7. In these blots the upper (128 kDa, one asterisk) and lower (108 kDa, two asterisks) amphiphysin I immunoreactive bands appear as doublets.

Fig. 5

Amphiphysin I is present in normal and neoplastic human mammary tissues. Total protein homogenates of (a) human brain (10 μg), (b) human breast cancer cell line Hs578T (100 μg), (c) primary human breast tumor (100 μg), and (d-h) normal human mammary tissues (100 μg each) were probed by western blotting using a monoclonal antibody directed against domain V (see fig. 1B) of amphiphysin I. One and two asterisks correspond to the 128 kDa and the 108 kDa amphiphysin I immunoreactive bands, respectively.

Fig. 6

Expression levels of amphiphysin I in normal and neoplastic human mammary tissues. Panel A: total protein homogenate of (a) human brain (10 μg), (b) Hs578T cell line (100 μg) , (c-e) normal human mammary tissues (100 μg each), (d-m) human primary human breast tumors (100 μg each), and (n) breast tumor from patient #692 (75 μg) were probed by western blotting with an anti-amphiphysin I monoclonal antibody. For reference, the same samples in fig. 5 lanes c and d appear in fig. 6 lanes g and c respectively. Panel B: the same blot as in B was probed with ^{125}I labeled protein A and a monoclonal antibody directed against the intermediate filament protein vimentin (arrow head) to control for total protein loading. One and two asterisks correspond to the 128 kDa and the 108 kDa amphiphysin I immunoreactive bands, respectively.

Fig. 7

Expression of amphiphysin I immunoreactivity in normal rat and human tissues. Total proteins of tissue homogenates were loaded in each lane and probed with monoclonal antibodies directed against domain V of amphiphysin I (see fig. 3). Protein loaded was as follows: rat tissues, 100 μgs/lane; human tissues, 10 μgs of brain homogenate, 100 μgs of the breast cancer cell line Hs578T and 100 μgs of all other tissue homogenates. One and two asterisks point to the 128 and the 108 kDa bands respectively.

Fig. 8

The 108 kDa amphiphysin I band contains a functional SH3 domain. Affinity-chromatography of a Triton X-100 extract from the Hs578T cell line onto GST-fusion proteins. The following material was probed by western blotting with monoclonal antibodies directed against amphiphysin I: (a), starting extract; (b), material affinity-purified on the full length proline-rich domain of dynamin I; (c), material affinity-purified

on a truncated proline-rich domain of dynamin I missing the amphiphysin I-binding site (construct DynPRD 751-832 of ref. 22); (d), material affinity-purified on GST alone.

Fig. 9

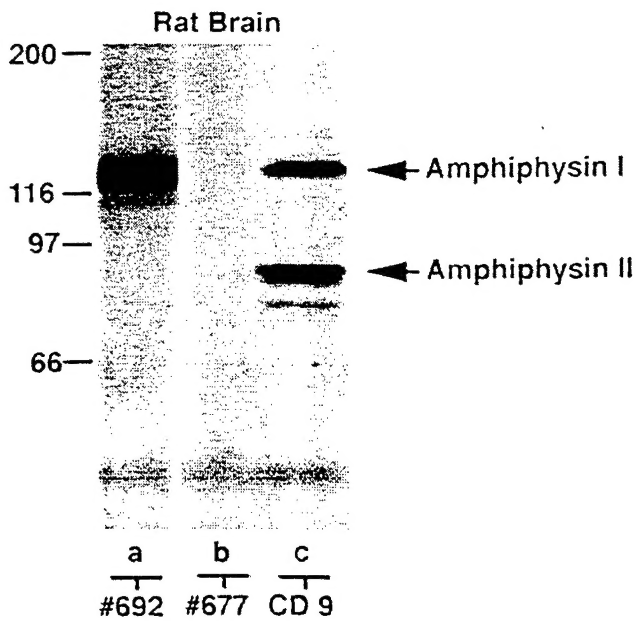
Sequence comparison of amphiphysin I clone 3.4 from Hs578T cell line and human brain amphiphysin I. The schematic drawing illustrates the amino acid differences between the human brain isoform of amphiphysin I and the isoform encoded by clone 3.4 from a cDNA library of the Hs578T breast cancer cell line. This 42 amino acid deletion was also detectable by RT-PCR in normal human kidney, heart and mammary tissues, as well as in at least two of the breast cancers with high level amphiphysin I immunoreactivity in fig. 6 above.

Fig. 10

In-vitro transcription/translation of amphiphysin I clone 3.4. The following material was probed by western blotting with a C00H-terminal directed anti-amphiphysin I monoclonal antibody: (a) human brain total protein homogenate, (b) Hs578T cell line total protein homogenate, (c) in-vitro transcribed/translated product of clone 3.4, (d) in-vitro transcribed/translated product of the luciferin cDNA, and (e) the product of a an in-vitro transcription/translation reaction in which no DNA was included.

FIGURE 1

A



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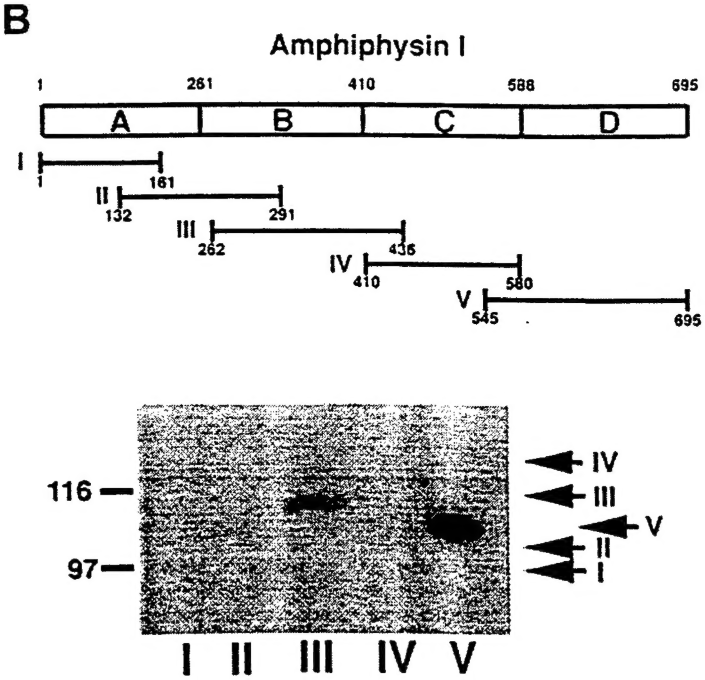

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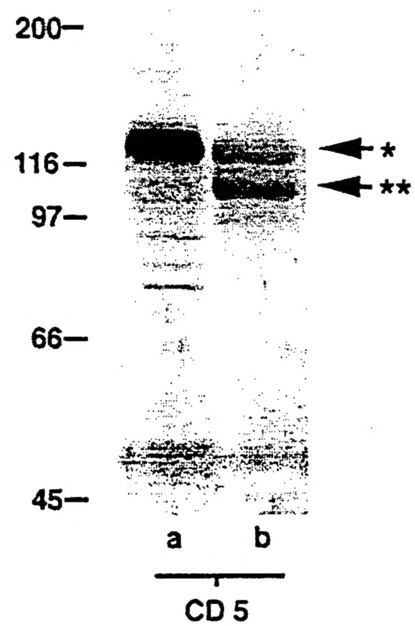

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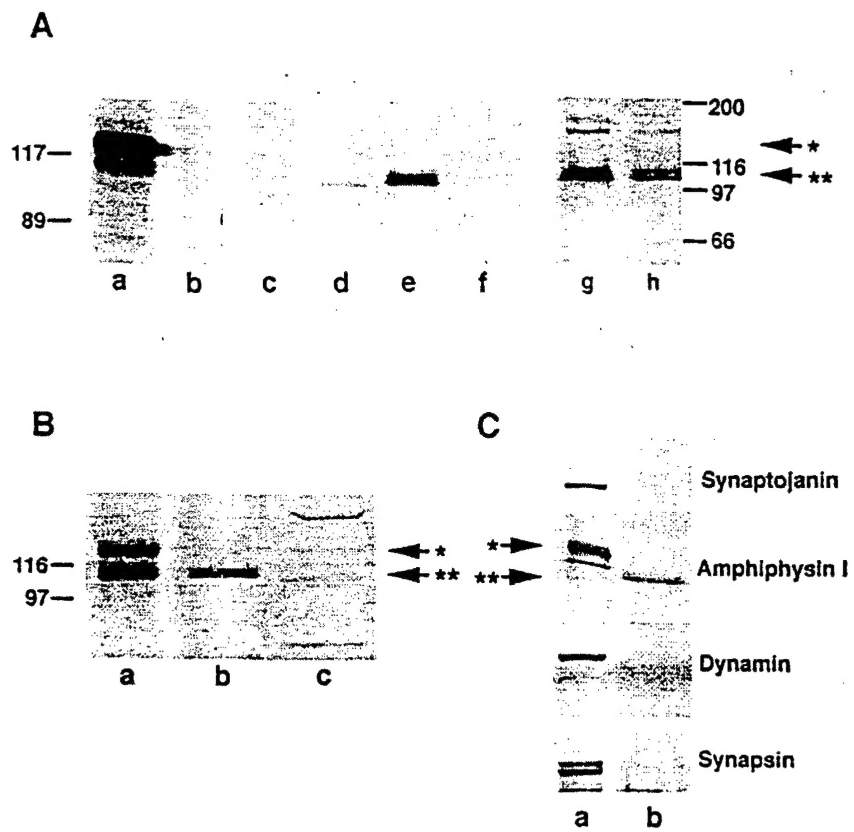

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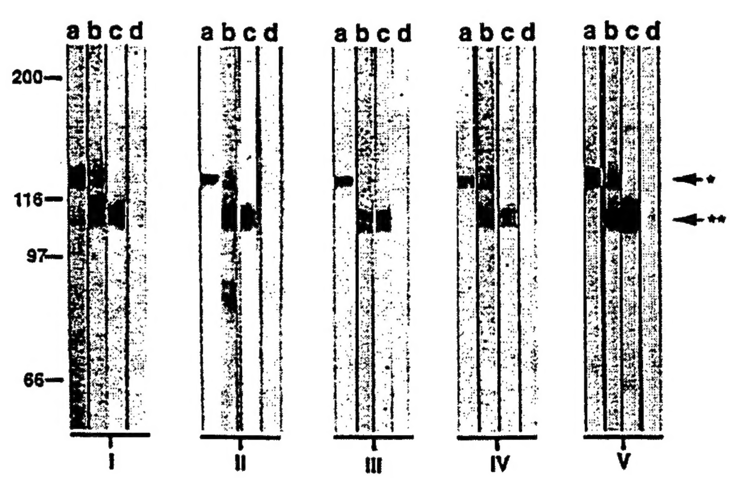

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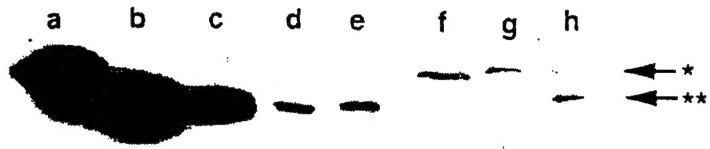

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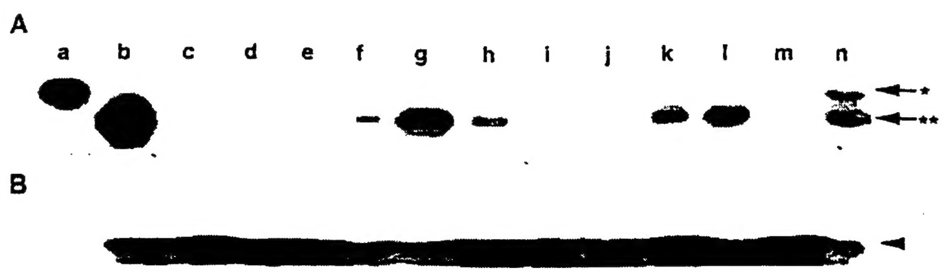

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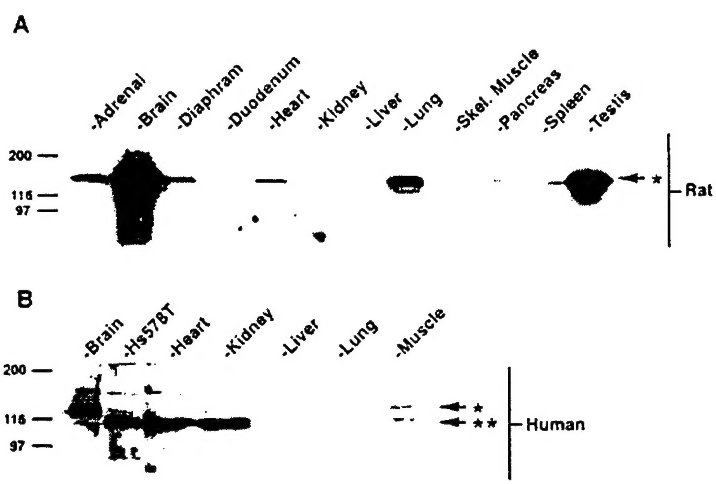

FIGURE 8

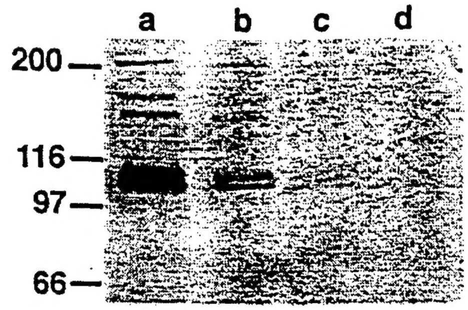

FIGURE 9

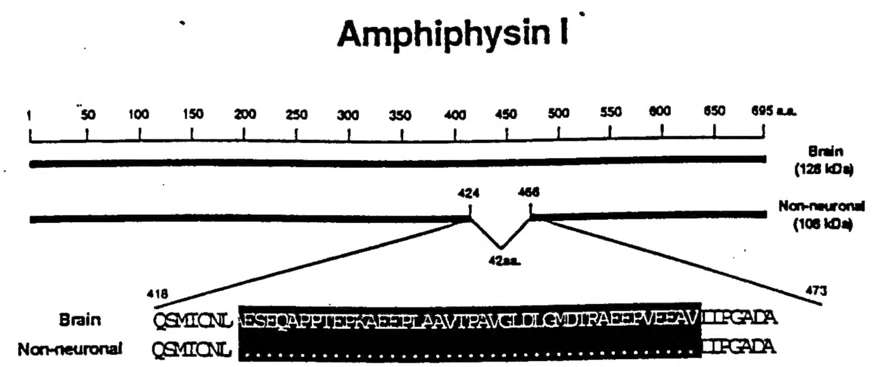

FIGURE 10